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RESEARCH PAPER

High-throughput HPLC–MS/MS determination of the persistence of neonicotinoid insecticide residues of regulatory interest in dietary bee pollen

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Abstract The aim of this work is the development of a simple, fast, quantitative, and economic method for the determination of neonicotinoid insecticide residues in dietary bee pollen. Several parameters of the method, such as extraction solvent, extraction time, and solid-phase extraction sorbents for purification [silica, C_{18}, primary–secondary amine (PSA), and Envi-Carb II/PSA], were studied. The final proposed method based on solid–liquid extraction with hexane, cleanup with Supelclean™ Envi-Carb II/PSA cartridges, and subsequent analysis by high-performance liquid chromatography with tandem mass spectrometry was validated and applied to the analysis of commercial bee pollen samples from different geographical zones. Method performance was assessed by the evaluation of several quality parameters of the method, such as recovery values, repeatability, reproducibility, linearity, and limits of detection and quantification. Matrix effects on the chromatographic signal were also studied. The quality parameters of the method were equivalent to or better than those obtained with previously published methods, with recoveries between 81 and 99 % and repeatabilities lower than 8.8 %. The detection and quantification limits were in the ranges 0.2-2.2 μg kg^{-1} and 0.4-4.3 μg kg^{-1}, respectively.

Keywords Dinotefuran · Nitenpyram · Thiamethoxam · Clothianidin · Imidacloprid · Acetamiprid · Thiacloprid · Pollen

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Introduction

Neonicotinoids belong to three chemical families: the *N*-nitroguanidines, nitromethylenes, and *N*-cyanoamidines [1, 2]. They can be used as a seed dressing [3]. They are often used as foliar sprays and as granular formulations for soil-dwelling insects [4]. It has recently emerged that neonicotinoids can persist and accumulate in soils [5]. When the treated seed is sowed, a small proportion is lost as dust [6]. This aerial dust can cause direct death of honeybees flying nearby [6, 7]. Neonicotinoids affect acetylcholine, causing lack of coordination, paralysis, and loss of sense of direction and effectiveness of their flight and may even lead to death [8, 9].

The impacts of neonicotinoids on bees aroused social interest promoted by beekeepers, which resulted in numerous press releases. In response to this concern, the European Commission asked the European Food Safety Authority to study their safety; the conclusion was that some of these insecticides could pose a high risk to bees. As a consequence, in 2013 Implementing Regulation (EU) No 485/2013 [10] came into force; this prohibits the use of three insecticides (clothianidin, thiamethoxam, and imidacloprid) both in seed and soil treatments and in crops attractive to bees and cereals, except for their uses in greenhouses and in winter cereals. Because of this, the development of simple, rapid, and economic methods for the determination of these compounds is mandatory.

Neonicotinoid residues were found by other authors in different matrices. In 2010, Mullin et al. [11] analyzed samples of bee pollen, and found acetamiprid and imidacloprid residues above the maximum residue limits (MRLs). In 2012, Phororecka et al. [12] found residues of clothianidin, thiacloprid, and thiamethoxam above the MRLs in some samples of nectar, and the levels of thiacloprid were well above the MRL in pollen samples. That same year, Stoner and Eitzer [13] found traces of thiamethoxam in pollen samples above

the MRL. In 2013, Giroud et al. [14] found residues of acetamiprid above the MRL in bee bread. More recently, Jovanov et al. [9, 15] assessed the contamination of honey liqueur and honey samples by neonicotinoids. Samples contaminated with levels below the MRLs but in some cases very close to the MRLs were found.

The use of chromatographic techniques to determine the selected neonicotinoids has become widespread in recent years, mainly because of their separating capacities, especially when selective detection techniques are used. Neonicotinoids in food (fruits and vegetables, pollen, honey, and other bee products) and agricultural (nectar, flowers, and grass) samples are usually determined by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) [9, 16–20] because direct analysis by gas chromatography [21, 22] is more complex owing to the low volatility and high polarity of these compounds. Traditional liquid–liquid extraction [23], solid-phase extraction (SPE) [16, 24, 25], matrix solid-phase dispersion [26], combinations of liquid–liquid extraction and SPE [13, 23], and dispersive liquid–liquid microextraction [16, 27] are the pretreatment procedures most commonly used for the analysis of neonicotinoid insecticide residues in food matrices such as honey and pollen.

The principal problems associated with the determination of neonicotinoid insecticide residues in dietary bee pollen are the low detection levels required and the diversity of potential interferences present in the sample matrix. Bee pollen is a very complex matrix that is a particular analytical challenge for pesticide residue analysis. It is mainly composed of proteins, lipids, sugars, fiber, mineral salts, amino acids, phenolic compounds, and vitamins. A high concentration of reducing sugars, essential amino acids, and unsaturated/saturated fatty acids, the presence of Zn, Cu, and Fe, and a high K/Na ratio make honey bee pollen very important for the human diet [28]. Therefore, the aim of this work was to develop and validate a multiresidue method for the determination of seven neonicotinoids (dinotefuran, nitenpyram, thiamethoxam, clothianidin, imidacloprid, acetamiprid, and thiacloprid) in dietary bee pollen samples. This method was then applied to the analysis of commercial bee pollen samples in order to evaluate their compliance with the MRLs established in EU legislation [29].

Materials and methods

Standards and reagents

Analytical standards of imidacloprid (purity 99.9 %), thiamethoxam (99.6 %), clothianidin (99.9 %), acetamiprid (99.9 %), thiacloprid (99.9 %), dinotefuran (99.0 %), and nitenpyram (99.9 %) were obtained from Sigma-Aldrich (Seelze, Germany). To prepare individual stock solutions at concentrations of 1000 mg L^{-1}, approximately 0.010±0.001 g of the analyte was added to a 10-mL volumetric flask and methanol was added to the correct volume (Sigma-Aldrich). Stock solutions were stored in amber glass bottles at −20 °C, and they were stable over a period of at least 3 months. Mixed working standard solutions were prepared daily by the appropriate mixture and dilution of the individual stock solutions in water or acetonitrile, as necessary. These solutions were used for spiking pollen samples, for matrix-matched calibration, and for solvent-based calibration. We prepared matrix-matched standards by extracting blank pollen samples and spiking them with the working solutions in the final reconstitution step.

Ultra gradient HPLC-grade water, acetonitrile for liquid chromatography (LC), and hexane for extraction were also purchased from Sigma-Aldrich. Other reagents used were magnesium sulfate anhydrous QP (purity 96 %) from Panreac (Barcelona, Spain) and sodium acetate from Sigma-Aldrich.

Small apparatus and disposables

For solid–liquid extraction, samples were placed in 50-mL polypropylene centrifuge tubes from Sterilin (Newport, UK), and they were shaken first with a Heidolph (Cinnaminson, NJ, USA) Reax top test tube shaker, then in a Branson (Carouge, Switzerland) 5800 ultrasonic bath, and finally in a Heidolph (Schwabach, Germany) Unimax orbital shaker. Polypropylene tubes were centrifuged in a Rotina 35 R centrifuge from Hettich Lab Technology (Tuttlingen, Germany), and the organic extracts were concentrated in a TurboVap LV concentration workstation (Caliper Life Sciences, Barcelona, Spain) with use of nitrogen C-50 purchased from Carburos Metálicos (Vigo, Spain). For sample purification, several sorbents were tested. Strata SI-1 silica cartridges (55 μm, 70 Å, 500 mg, 6 mL) and Strata C18-T cartridges (55 μm, 140 Å, 2 g, 12 mL) were from Phenomenex (Utrecht, The Netherlands). Supelclean™ Envi-Carb II/primary–secondary amine (PSA) (500 mg, 6 mL) SPE tubes and dispersive SPE Supelclean™ PSA cleanup Tube-1 (50 μm, 70 Å, 6 mL) were from Supelco (Bellefonte, PA, USA).

Final organic extracts were filtered through Chromafil® Xtra PET-20/25 (0.20-μm) filters from Macherey-Nagel (Düren, Germany) and placed in 2-mL amber vials from Supelco before chromatographic analysis.

Analytical pretreatment

Commercial bee pollen samples from different regions of Spain were purchased from different markets in Ourense (northwest Spain). All samples were kept in their original packaging at room temperature. For method optimization and validation, an uncontaminated bee pollen sample from Toledo (Spain) was used as a blank sample. Aliquots of the

sample (5 g±0.1 g) were fortified with working standard solutions of all pesticides at different levels in acetonitrile. Samples were then left in the dark (15 min) for stabilization.

For neonicotinoid determination, samples were extracted according to the following procedure. An aliquot (5 g) of bee pollen previously ground in a mortar was weighed in a 50-mL polypropylene centrifuge tube. Then 10 mL of pure water, 10 mL of hexane, 10 mL of acetonitrile, 6 g of magnesium sulfate, and 3 g of sodium acetate were added to the tube. The mixture was then vigorously shaken in a test tube shaker (1 min), treated for 10 min in an ultrasonic bath at room temperature, and finally shaken for 20 min in an orbital shaker at 200 rpm. The mixture was then centrifuged at 5000 rpm and 10 °C for 10 min. An aliquot (7 mL) of the supernatant was transferred to a 15-mL tube and treated with a small amount of magnesium sulfate (around 1 mg) to remove water. Subsequently, the extract was centrifuged again (4000 rpm, 10 °C for 3 min), and 6 mL of the supernatant was passed through a Supelclean™ ENVI-Carb II/PSA cartridge previously conditioned with 6 mL of acetonitrile. The eluent was collected, and the cartridge was rinsed with 3 mL of acetonitrile. The combined eluents were evaporated to dryness in a water bath under a stream of nitrogen. The extract was then reconstituted to 0.75 mL with water, filtered through a 0.20-μm filter, and analyzed by HPLC–MS/MS.

HPLC–MS/MS conditions for detection and quantification

HPLC analyses were performed with a Thermo Surveyor HPLC system equipped with a LC Pump Plus, a Plus Lite autosampler, a TSP SCM1000 vacuum membrane degasser, and a Gecko 2000 column heater from Cil Cluzeau Info Labo (France) linked to a PC running Xcallibur version 5.0. A Hypersil GOLD™ column (100 mm×4.6-mm inner diameter; 5 μm) with a Hypersil GOLD™ drop-in guard cartridge (10 mm×4.6-mm inner diameter, 5 μm) from Thermo Scientific (Waltham, MA, USA) was used.

After different mobile phase systems and gradients had been assayed, an acetonitrile–HPLC water mobile phase at a flow rate of 1.0 mL min^{-1} was selected. The target neonicotinoids were separated with the following gradient program: 90 % water for 2 min, followed by a linear gradient to 70 % water within 4 min, 60 % water in 4 min, 5 % water within 1 min, 5 % water for 2 min, and then the initial conditions were recovered within 2 min. A postrun time of 15 min was set for column equilibration, giving a total run time of 30 min.

The HPLC system was coupled to a TSQ Quantum Discovery triple-stage quadrupole mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA). The mass spectrometer was operated in positive electrospray ionization mode under the following specific conditions: spray voltage of 3200 V, capillary temperature of 250 °C, sheath gas and auxiliary gas pressure of 60 and 0 units, and peak width of 0.7 (full width at half maximum). For each neonicotinoid, the protonated molecular ion $[M+H]^+$ was chosen as the precursor. Two multiple reaction monitoring transitions were optimized. The target ion transition with the highest intensity was used for quantitation, whereas the second transition was used for confirmation. The chemical structure, retention time, ion transitions, dwell time, and collision energy for each compound are displayed in Table 1. The retention times were constant, with the relative standard deviation (RSD) never exceeding 0.06 %. In addition, no interfering peaks were observed at the retention times of neonicotinoids in the blank pollen.

Method performance

The performance of the HPLC–MS/MS method was assessed in accordance with the recommendations of the CITAC/EURACHEM guidelines [30] and SANCO document no. SANCO/12495/2011 [31]. The fundamental parameters assessed were recovery, repeatability, reproducibility, linearity, limits of detection (LODs), and limits of quantification (LOQs). Matrix effects were also evaluated.

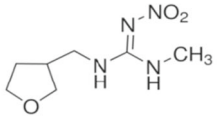
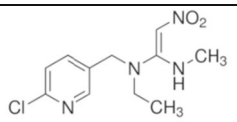
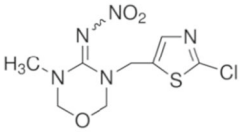
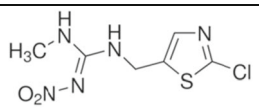
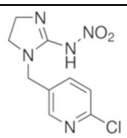
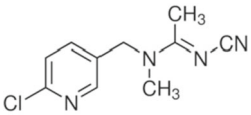
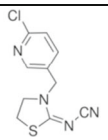
Calibration curves for neonicotinoids in pure solvent and in pollen matrix were obtained by plotting the analyte areas against the concentrations of the corresponding calibration standards at six levels. The linearity of the calibration curves was expressed by the squared correlation coefficient (r^2). In addition, a linearity test was done with the statistical software package Statgraphics version 15.2.05 (Manugistics, Rockville, MD, USA) to evaluate the quality of the fit.

Matrix-induced suppression/enhancement effects were evaluated by comparing the chromatographic signal obtained for the direct injection of standards of the target neonicotinoids in water (solvent-based calibration) with that of the matrix-matched calibration obtained after extraction of a blank bee pollen sample spiked with neonicotinoids in the final reconstitution step. Recoveries were determined using blank pollen samples (n=5) spiked before analysis at levels of 12.5 μg kg^{-1} and matrix-matched calibration curves by comparing the analyte content determined from the curve with that added to the sample.

Repeatability was assessed by analysis of five blank pollen samples spiked (12.5 μg kg^{-1}) on the same day with the same instrument and the same operator, whereas reproducibility was estimated by analysis of nine spiked blank pollen samples on 3 days in two different weeks with the same instrument and different operators.

LODs and LOQs were evaluated on the basis of the signal obtained in the analysis of blank pollen samples (n=5). The LODs and LOQs were the analyte concentration whose response is equivalent to the mean blank response plus three or ten times the standard deviation, respectively.

Table 1 Chemical structure, mean retention time (*RT*), tandem mass spectrometry (MS/MS) transitions for quantification (*MRM1*) and confirmation (*MRM2*), dwell time, and collision energy used for each compound

Compound	RT (min)	Transitions	Dwell time (s)	Collision energy (eV)	Chemical structure
Dinotefuran	4.4	MRM1 203.2→ 129.0 MRM2 203.2→ 157.1	0.05	12 8	
Nitenpyram	6.4	MRM1 270.6→ 125.6 MRM2 270.6→ 188.7	0.05	25 25	
Thiamethoxam	7.6	MRM1 292.2→ 211.1 MRM2 292.2→ 181.1	0.05	12 24	
Clothianidin	8.4	MRM1 250.2→ 169.0 MRM2 250.2→ 132.0	0.05	12 14	
Imidacloprid	8.7	MRM1 256.2→ 175.1 MRM2 256.2→ 209.2	0.05	18 14	
Acetamiprid	9.2	MRM1 223.2→ 126.1 MRM2 223.2→ 187.1	0.05	20 12	
Thiacloprid	10.3	MRM1 253.2→ 126.1 MRM2 253.2→ 186.2	0.5	20 12	

The validation criteria were as follows: regression coefficients higher than 0.99 for linearity, recoveries between 70 and 120 %, and RSDs lower than 20 % for repeatability and lower than 25 % for reproducibility.

Results and discussion

Optimization of the extraction process

The aim of this stage was to define the optimal conditions in terms of recovery for the extraction of the target neonicotinoids from bee pollen samples. Different parameters of the extraction technique, such as volume of the extraction solvent, extraction time, and SPE sorbent for sample purification, were optimized. All experiments were done in triplicate.

In the literature, some authors proposed a solid–liquid extraction with acidified acetonitrile as the extraction solvent, followed by purification of the extract with dispersive PSA before chromatographic analysis [32, 33]. In this way, the sample (2 g of blank bee pollen spiked with the target compounds at levels of 4 mg kg^{-1}) was extracted with 10 mL of acetonitrile with 1 % acetic acid for 2 min and centrifuged (8 min, 4,000 rpm, 10 °C). Then, the extract (6 mL) was treated with dispersive PSA, and finally injected into an HPLC–MS/MS system. The results obtained showed that insecticides were not extracted quantitatively. In addition, recoveries between 67 and 74 % were obtained when the method was applied to the extraction of aqueous solutions of these compounds at the same level. To improve the extraction recoveries, other resins for sample purification were tested: silica, C_{18}, and Supelclean™ Envi-Carb II/PSA. As the

objective of this step was to evaluate the purification efficiency of these sorbents, an unspiked bee pollen sample (2 g) was extracted as described above, and the extract was fortified with the neonicotinoids before the purification step. The spiked extract (6 mL) was then loaded into each cartridge previously conditioned with acetonitrile with 1 % acetic acid. The eluent was recovered, and the cartridge contents were eluted with 3 mL of acetonitrile with 1 % acetic acid. Although the analytes were not retained in the cartridge, some coeluted interferences were removed, and colorless extracts were obtained. The final extract was concentrated to dryness under a stream of nitrogen, and was redissolved in water before HPLC–MS/MS analysis. Unsatisfactory results were obtained with C_{18} and silica cartridges, with recoveries lower than 70 % for nitenpyram and imidacloprid and higher than 140 % for clothianidin. The best results were obtained with Supelclean™ Envi-Carb II/PSA cartridges, with recoveries ranging between 75 and 100 % for all the target compounds.

Another parameter that affects recoveries is the extraction time. To evaluate its influence, fortified bee pollen samples were extracted with acidified acetonitrile at different times (2, 15, and 30 min), and the extracts were purified with Supelclean™ Envi-Carb II/PSA cartridges. As shown in Fig. 1a, an increase of the extraction time from 2 to 15 min improved significantly the extraction yields, with recoveries ranging between 83 and 96 %. Although similar recoveries (87 and 103 %) were obtained with a longer extraction time (30 min), lower standard deviations were observed. Because of this, 30 min was selected as the optimum extraction time. However, under these conditions the method did not have enough sensitivity to achieve the restrictive MRLs established in the EU legislation.

The sensitivity of the method could be improved in two ways: increasing the concentration of neonicotinoids in the final extract by increasing the amount of sample; or with a better purification of the sample to decrease the number of interfering compounds. As a first attempt, the amount of pollen was increased from 2 to 5 g, and the sample was extracted with different volumes of acidified acetonitrile (10, 20, and 30 mL). As it can be seen in Fig. 1b, the best results were obtained for 30 mL, with recoveries ranging between 65 and 106 %. Nevertheless, with this sample-to-solvent ratio the sensitivity of the method did not improve. Therefore, this option was discarded.

Giroud et al. [14] proposed the use of low temperature and a nonpolar solvent to remove sugars, waxes, and lipids from the matrix and decrease its contribution to the chromatographic signal. Moreover, other authors proposed increasing the water content of the sample to facilitate the extraction of neonicotinoids [14, 17, 18]. In our case, the use of low

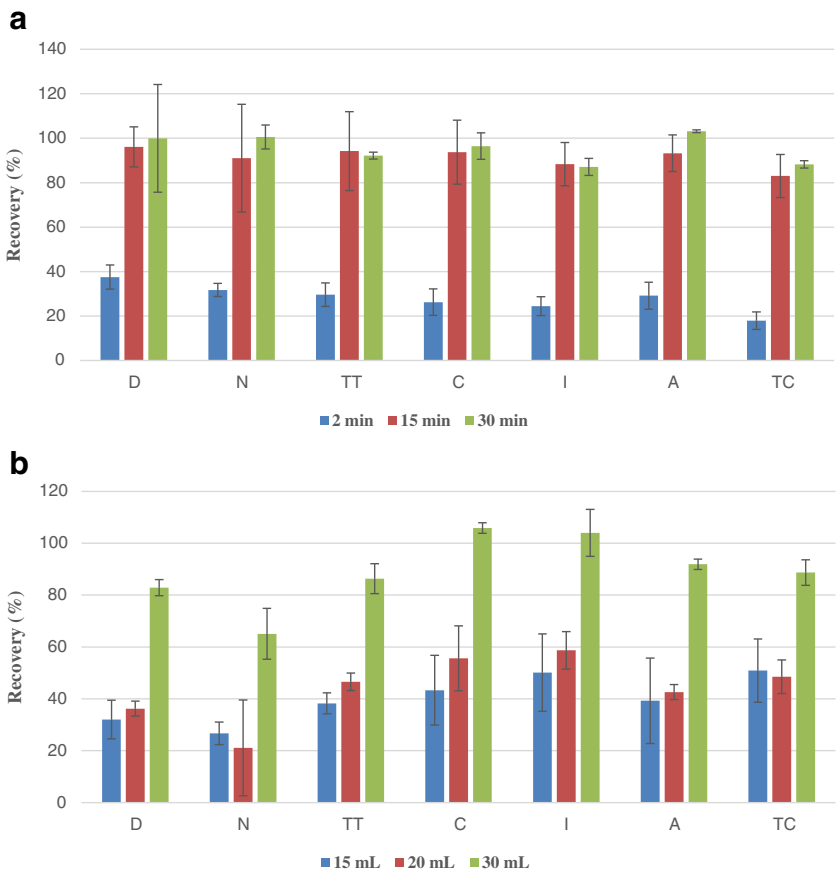


Fig. 1 Effect of the extraction time (**a**) and extraction volume of acidified acetonitrile (**b**) on the extraction efficiency (recovery± standard deviation) of neonicotinoids from bee pollen samples (n=3). *A* acetamiprid, *C* clothianidin, *D* dinotefuran, *I* imidacloprid, *N* nitenpyram, *TC* thiacloprid, *TT* thiamethoxam

temperature did not improve the signal. However, the addition of 10 mL of water and 10 mL of hexane resulted in a decrease in the number of coextracted compounds and an improvement of the sensitivity. In the final method, the use of acidified acetonitrile was replaced by the use of 100 % acetonitrile, since the acidification of the extraction solvent is crucial for the determination of some neonicotinoid metabolites [14] but not for the parent compounds, as found experimentally. A total ion chromatogram obtained for the analysis of a blank pollen sample spiked with the target neonicotinoids under the optimized conditions is shown in Fig. 2.

Analytical performance

Several quality parameters of the method, such as recoveries, repeatability, reproducibility, linearity, LODs, LOQs, and matrix effects, were evaluated as described in "Method performance".

Matrix effects, expressed as the signal from the neonicotinoids in the matrix compared with the signal in the solvent, were calculated as signal suppression/enhancement. As it can be seen in Table 2, pollen matrix leads to an important ion suppression of the analyte. Matrix effects were also found in pollen matrices by other authors [34]. Therefore, to overcome this problem and to achieve precise quantification, matrix-matched calibration was used as recommended in the EU guidelines [31].

The matrix-matched calibration curves for the neonicotinoids were linear over the range from the LOQ to 62.5 μg kg^{-1}, with correlation coefficients (r^2) higher than 0.995, as shown in Table 2. In addition, a linearity test was done to evaluate the quality of the fit. The sum of squared deviations obtained for each compound ranged between 9.7×10^{9} and 2.9×10^{12}. *P* values lower than 0.05 were obtained in all cases, indicating that there is a statistically significant relationship between analyte area and concentration for a confidence level of 95 %.

The recoveries were between 81 and 99 % (Table 3), with RSDs lower than 9 %, showing the good accuracy of the method according to the EU validation guidelines for pesticides [31]. LODs of about 0.2-2.2 μg kg^{-1} and LOQs of about 0.4-4.3 μg kg^{-1} were obtained, as summarized in Table 3. The LOQs obtained were lower than the MRLs established in the EU legislation for neonicotinoids in pollen samples. The RSDs obtained for repeatability and reproducibility show the good intraday (RSD<9 %) and interday (RSD<14 %, except for nitenpyram) precision of the method (Table 3).

The quality parameters of the proposed method are equivalent to or better than others reported in the literature, as shown in Table 3. The proposed method produces better recoveries (more than 81 % for all compounds) and LOQs (less than 1.6 μg kg^{-1}) than the method proposed by Pohorecka et al. [12] (recoveries between 70 and 110 % and LOQs between 1 and 3 μg kg^{-1}). Although similar recoveries (87-111 %) were obtained by Chen et al. [35] and Kamel [17], the proposed method has better precision (RSD<9 % for all compounds).

Application to dietary bee pollen

Different samples of commercial bee pollen from diverse geographical zones of Spain, as indicated on the label, were analyzed. In general, no residues of these compounds were found above the LOQs, except in one sample from Valencia, where residues of acetamiprid and imidacloprid at levels of 9 and 13 μg kg^{-1}, respectively, were found. Those levels are five times lower than the levels established in the EU legislation for pollen samples [29]. Residues of thiacloprid below its

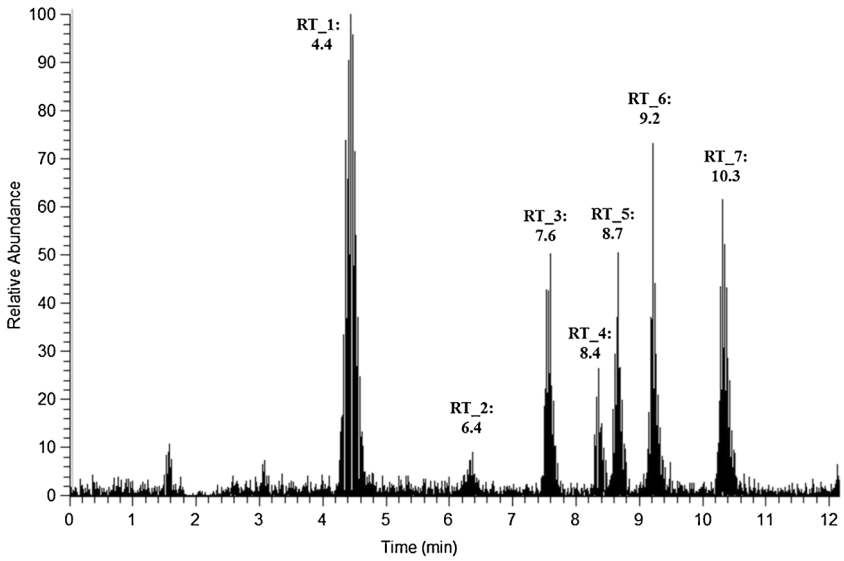


Fig. 2 Total ion chromatogram corresponding to a blank bee pollen sample spiked with a mixture of the seven neonicotinoids at 25 μg kg^{-1}. *RT_1* dinotefuran, *RT_2* nitenpyram, *RT_3* thiamethoxam, *RT_4* clothianidin, *RT_5* imidacloprid, *RT_6* acetamiprid, *RT_7* thiacloprid

Table 2 Matrix effects calculated as the slope ratio of matrix-matched calibration and solvent-based calibration curves for selected neonicotinoids

Neonicotinoid	Matrix-matched calibration		Solvent-based calibration		SSE (i.e., slope ratio, %)
	Linear equation	Correlation coefficient (r^2)	Linear equation	Correlation coefficient (r^2)	
Dinotefuran	y=8151623.38x+58675.45	0.9978	y=14196473.36x – 28921.77	0.9999	57.42
Imidacloprid	y=1929552.59x+27335.31	0.9963	y=4117837.57x – 25024.49	0.9997	46.85
Thiamethoxam	y=2751862.35x+38360.38	0.9953	y=5803972.94x – 25995.49	0.9999	47.41
Acetamiprid	y=2233173.51x+37689.28	0.9984	y=7690623.71x+3034.61	0.9998	29.03
Clothianidin	y=1166430.70x+4322.75	0.9971	y=2426587.79x – 14428.10	0.9996	48.06
Thiacloprid	y=2700824.50x+121297.07	0.9972	y=6103589.79x+16,824.40	0.9991	44.24
Nitenpyram	y=563157.13x+7750.81	0.9984	y=750307.56x – 9855.15	0.9998	75.06

SSE signal suppression/enhancement

LOQ were found in the same sample from Valencia and in a pollen sample from Madrid. The results obtained are on the same order as those found by other authors, as shown in Table 4. As it can be seen in this table, thiacloprid, acetamiprid, and imidacloprid are the neonicotinoids most frequently detected, as occurred in this study.

Table 3 Quality parameters of the proposed method

The proposed method: HPLC–MS/MS method (SLE and Envicarb II/PSA cleanup)						
Analyte	Recovery (%)	Repeatability (RSD, %; n=5)	Reproducibility (RSD, %; n=9)	LOD (μg/kg; n=5)	LOQ (μg/kg; n=5)	MRL (μg/kg)
Dinotefuran	96.9	1.6	8.8	0.7	0.9	10
Imidacloprid	81.5	8.8	10.8	0.6	1.2	50
Thiamethoxam	97.4	5.7	8.4	0.7	1.6	10
Acetamiprid	92.4	2.3	11.9	0.4	0.9	50
Clothianidin	96.6	2.0	13.4	0.6	1.3	10
Thiacloprid	88.6	3.5	10.0	0.2	0.4	200
Nitenpyram	98.9	7.7	19.5	2.2	4.3	10
Methods proposed by other authors						
Analyte	Sample	Method	Recovery±precision (%)	LOD (μg/kg)	LOQ (μg/kg)	Reference
Imidacloprid	Pollen and bee bread	SLE–LC–MS/MS	70-110	0.8	1.5	[12]
Thiamethoxam				0.3	3.0	
Acetamiprid				0.2	1.0	
Clothianidin				1.0	3.0	
Thiacloprid				0.4	2.0	
Dinotefuran	Bee pollen	SLE–LC–MS/MS	87.2±9.7	0.2	-	[17]
Imidacloprid			108.2±13.2	0.2	-	
Thiamethoxam			110.5±12.8	0.2	-	
Clothianidin			111.9±5.5	1.0	-	
Dinotefuran	Pollen	QuEChERS–LC–MS/MS	96.1±20	-	0.1	[35]
Imidacloprid			101.6±12	-	0.1	
Thiamethoxam			101.5±10	-	0.1	
Acetamiprid			110.4±13	-	0.1	
Clothianidin			97.5±14	-	0.1	
Thiacloprid			99.6±17	-	0.1	
Nitenpyram			97.4±18	-	0.5	

HPLC high-performance liquid chromatography, *LOD* limit of detection, *LOQ* limit of quantification, *MRL* maximum residue limit, *PSA* primary–secondary amine, *QuEChERS* quick, easy, cheap, effective, rugged, and safe, *RSD* relative standard deviation, *SLE* solid–liquid extraction

Table 4 Determination of neonicotinoid residues in commercial bee pollen samples

Country	Analyte	No of samples	Positives	Content (μg kg^{-1})	Reference
Spain	Acetamiprid	5	1	9.0	This work
	Clothianidin	5	0	0	
	Dinotefuran	5	0	0	
	Imidacloprid	5	1	13.0	
	Nitenpyram	5	0	0	
	Thiacloprid	5	2	<LOQ	
	Thiamethoxam	5	1	<LOQ	
	Acetamiprid	27	1	5.8	[36]
	Imidacloprid	27	1	5.2	
	Imidacloprid	1021	0	0	[37]
	Imidacloprid	61	0	0	[38]
Canada	Clothianidin	4	4	0.1-0.8	[39]
	Thiamethoxam	4	0	0	
Germany	Imidacloprid	215	1	3	[40]
	Thiacloprid	215	9	<199	
	Clothianidin	215	0	0	
	Acetamiprid	215	2	0	
Greece	Acetamiprid	19	6	6.1-1273	[34]
	Clothianidin	3	1	72-73.9	
New Zealand	Acetamiprid	6	0	0	[35]
	Clothianidin	6	6	0.2-1.9	
	Dinotefuran	6	0	0	
	Imidacloprid	6	5	0.2-1.2	
	Nitenpyram	6	0	0	
	Thiacloprid	6	6	0.1-3.3	
	Thiamethoxam	6	0	0	
Poland	Acetamiprid	205	45	4.1-26.1	[12]
	Clothianidin	205	11	1.8-3.7	
	Imidacloprid	205	0	0-0	
	Thiacloprid	205	62	89.1-1002.2	
	Thiamethoxam	205	37	3.8-9.9	
USA	Acetamiprid	6	0	0	[35]
	Clothianidin	6	0	0	
	Dinotefuran	6	0	0	
	Imidacloprid	6	5	0.4-2.3	
	Nitenpyram	6	0	0	
	Thiacloprid	6	0	0	
	Acetamiprid	350	11	14-134	[11]
	Imidacloprid	350	10	6.2-206	
	Thiacloprid	350	19	1.7-115	
–	Imidacloprid	12	-	6-28	[13]
	Thiamethoxam	12	-	5-35	

Conclusions

The determination of neonicotinoids in bee pollen was performed in a short time and proved not to affect the stability of the compounds. Good recoveries (higher than 81 %) and precisions (below 9 %) were obtained for all compounds. The LODs and LOQs for the neonicotinoid insecticide residues in bee pollen were found to be satisfactory and much lower than the restrictions given in EU legislation for pollen samples destined for human intake. This method is recommended for the determination of residues of the seven neonicotinoid insecticides in bee pollen samples, since it was proved to be fast, easy, accurate, linear, and able to detect trace amounts of these compounds in the samples far below the levels set in any

potential legislation. The analysis of different samples of bee pollen of different origin revealed that in most of the samples, none of the pesticides studied were found. Only acetamiprid and imidacloprid were found in one of the samples at levels much lower than the corresponding MRL established by EU legislation. The analytical method developed could eventually be used for studies of these and similar insecticides in other foods.

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